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(54) Title: RETROVIRAL GENE TRANSFER VECTORS

CONSTRUCTION OF pSAM

(57) Abstract

Recombinant DNA constructs include 5' and 3' retroviral LTR sequences, a genome packaging sequence, a promoter sequence and a eukaryotic cDNA sequence under the promotional control of the promoter sequence. By making the retroviral sequences of the construct deficient in coat protein-encoding sequences, the construct is by itself incapable of packaging its genome as a retrovirion. The promoter sequence may be a viral LTR sequence. Alternatively the promoter sequence may be a nonviral promoter linked to the cDNA sequence, promoting the cDNA irrespective of cellular mechanisms which affect viral sequence expression. The retroviral genomes corresponding to the constructs are rescued as complete packaged virions by helper virus vectors which encode the coat proteins that are needed to package the genomes corresponding to the constructs. Helper virus vectors that are deficient in packaging sequences do not package their own genomes, and cells incorporating such a deficient helper virus vector and also a construct shed only retrovirions that are infectious a single time. Helper viral vectors which produce amphotropic proteins package recombinant retrovirions that are infective of cells of a variety of species. Cells infected with recombinant retrovirions are transplantable into an animal to comprise a portion of the somatic cells of the animal and express recombinant gene product therein. Recombinant retrovirionsaccording to the invention, if inoculated into the peripheral fluid of a host animal, will infect and genetically alter the somatic cells of the animal.

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RETROVIRAL GENE TRANSFER VECTORS

The present invention relates generally to the
use of retroviral vectors for transferring genetic
information into cells.

5   ## BACKGROUND OF THE INVENTION

The ability to introduce eukaryotic genes into
eukaryotic cells has contributed much to our current
knowledge of the mechanism and regulation of gene
expression. A variety of methods have been used to
10   transfer foreign genetic material into cells, each
method having certain advantages and limitations.

The widely used calcium phosphate-mediated
gene-transfer (transfection) technique is relatively
inefficient and is limited to use in cultured cells.
15   Introduction of foreign sequences by microinjection into
target cells is efficient but requires sophisticated
instrumentation.

An alternative approach for transferring genes
into cells involves the use of viral vectors. The
20   commonly used papovavirus vectors, such as simian virus
40 (SV40), however, have major limitations: (i) the
length of the foreign DNA segment cannot exceed 2.5
kilobase pairs (kbp) and (ii) papovaviruses have a
limited host range.

25   Because of their unique structure and mode of
propagation, retroviruses (a class of viruses having a
single-stranded RNA genome and a particular life cycle)
appear to be ideally suited as gene-transfer vehicles.
Some of their unique properties include (i) the RNA
30   viral genome is efficiently transmitted to the recipient
cells and integrated into the chromosome(s) as
double-stranded DNA, (ii) integration is specific with
respect to the viral genome, (iii) the plasticity of the
viral genome permits packaging of DNA inserts of up to
35   at least about 7 kbp, (iv) retroviruses have a wide host
range and can infect a variety of cell types, and (v)
viral long terminal repeats (LTRs) provide efficient

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signals for initiation and termination of
transcription. Several groups have recently used
retroviral DNA vectors to generate infectious virus
containing either the herpes simplex virus thymidine

5   kinase (TK) gene or the Escherichia coli guanine
phosphoribosyltransferase gene (Eco gpt).
    It is of particular interest to use
retroviruses for imparting genetic characteristics to
vertebrate cells, including human cells for a variety of

10 purposes. A genetic disease might be corrected, for
example, by introducing genetic material into a
sufficient population of somatic cells of a living
animal, including a human. A valuable gene-encoded
product, such as a pharmaceutically useful peptide,

15 might be farmed in a host animal. Animals that are
raised as a source of food might be genetically altered
so as to be more efficient protein producers. Desirable
characteristics of a retrovirus that is to be used for
introducing characteristics into eukaryotic cells

20 include flexibility that allows the retrovirus to be
used for introducing genetic characteristics into a wide
variety of cell types from a variety of species, the
inability of the retrovirus to cause a spreading
infection or to otherwise bring about deleterious

25 changes in the infected cell or in the animal as a
whole, efficiency of gene transfer into cells, and
efficient encoding of the desired genetic characteristic
carried by the retrovirus.

SUMMARY OF THE INVENTION

30   Novel retroviral vectors are constructed and
used to carry genetic information to the cells through
infection. DNA constructs are initially assembled from
proviral DNA sequences (DNA sequences corresponding to
RNA sequences of the retroviral genome) as well as DNA

35 sequences obtained from nonviral sources, including
genes encoding proteins that, when expressed, impart
novel characteristics to the cells. The viral sequences

used in the constructs include long term repeating (LTR)
sequences which include sequences necessary for the
genome to insert itself in DNA form (proviral form) into
a cell's genome and also provide efficient signals for
5   initiation and termination of transcription. The
constructs also retain viral sequences that direct
packaging of viral genome RNA. In order to avoid
producing recombinant virions capable of generating a
spreading infection, in preferred embodiments of the
10   invention, regions encoding viral proteins are removed
and/or truncated.

The DNA constructs are introduced into cells by
conventional methods, such as calcium phosphate
transfection, and a gene product is expressed in the
15   cells. The constructs, if deficient in packaging
protein-encoding sequences, can be "rescued" as packaged
retrovirions from the cells by introducing into a
construct-transfected cell a "helper" retroviral
vector. These helper viral vectors express packaging
20   proteins within the cells that are necessary for viral
replication. The helper virus vector is rendered
defective by removing its own packaging sequences, so
that it produces the needed proteins to package the
construct-encoded RNA genome but does not package its
25   own genome.

It is also preferred that the helper retroviral
vector be rendered defective so that it cannot package
itself into the gene of the host cell. The rescued
retrovirion that is obtained from the construct is
30   efficiently infectious into eukaryotic cells, and the
packaged virus inserts its genetic information linearly
into the DNA of the host cell. However, lacking
necessary protein-encoding sequences, it is incapable of
repackaging itself and thereby induce a spreading
35   infection. The recombinant retrovirus constructs can be
used to carry nucleic acid chains of substantial length,
e.g., up to about 7 kilobase pairs (kbp), into cells,
and both selectable genes and nonselectable genes may be

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carried into cells. If the RNA genome corresponding to the construct is packaged with an amphotropic helper virus, the resulting recombinant retrovirion exhibits infectivity that crosses biological family and even
5   phylum lines.

      The recombinant retrovirions according to the invention may be used to infect vertebrate animal cells _in vitro_, and the infected cells may then be transplanted into an animal where the infected cells
10   express the protein product that is encoded by the genetic material that was carried to the cells by the retrovirion.

      Genetic material may be inserted into retroviral vectors under the control of the LTR viral
15   sequences. Alternatively, it is found that genes may be inserted into retroviral vectors under control of their own promoter sequences, in which case, they are expressed in cells irrespective of the degree of functionality of the viral sequences themselves within
20   the cells.

<div align="center">IN THE DRAWINGS</div>

      FIGURE 1 is a schematic representation of the assembly of DNA constructs, pLPAL and pLPL, which embody various features of the present invention;
25   FIGURE 2 is a schematic representation of the assembly of a helper virus construct, designated pSEM, embodying various features of the present invention;

      FIGURE 3 is a schematic representation of an amphotropic helper virus construct, designated pSAM,
30   embodying various features of the present invention;

      FIGURE 4a is a schematic representation of construction of a rat growth hormone mini-gene;

      FIGURE 4b is a map of the rat growth hormone mini-gene;
35   FIGURE 5a is a schematic representation showing insertion into the pLPL construct of FIG. 1 of the rat growth hormone mini-gene of FIGURE 4b; the mini-gene

being inserted in a manner such that expression of the
rat growth hormone gene is under the control of the
endogenous rat growth hormone gene promoter; and

FIGURE 5b is a map of the viral genome-encoding
5   portion of the construct of FIGURE 5a.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

DEFINITIONS

The following terms are defined below as they
are used in this application:

10   Retrovirus -- a virus having a single-stranded
RNA genome and having a life cycle described in
Kornberg, DNA Replication, W.H. Freeman & Co., San
Francisco (1980) pp. 596-598.

Provirus -- the double-stranded counterpart of
15   the retroviral genome that comprises an essential stage
of the life cycle of a retrovirus.

Virion -- a packaged, infectious viral genome,
including retroviral genomes that are packaged with
proteins that are not encoded by their own nucleic acid
20   sequences.

Construct -- a recombinant DNA assemblage
including viral sequences.

Retroviral vector -- a vehicle for introducing
genetic material into a cell that includes retroviral
25   sequences; the term is used herein to generally include
DNA and RNA material and in packaged or non-packaged
form.

Helper virus -- a virion capable of infecting a
cell and encoding replication protein and packaging
30   genomic RNA for further propagation when introduced into
a cell.

Helper virus vector -- a helper virus as
defined above, and including plasmid DNA containing the
viral gene which can be transfected into cells but
35   unable to propagate.

Production of gene-carrying, infectious,
recombinant retroviral vectors according to the present

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invention begins with the assembly of a DNA construct
that contains DNA sequences corresponding to RNA
sequences of a parent(s) retrovirus as well as
additional DNA sequences, usually including a DNA
5   sequence that encodes a desired protein product. The
DNA construct generally includes LTR viral sequences of
the parent retrovirus including 5' and 3' sequences that
are necessary for insertion of the genome in its
proviral form into the chromosomal DNA of the host
10   cell. Portions of the LTRs that signal initiation and
termination of transcription of protein encoding
segments may also be included in the construct,
depending upon its purpose. Importantly, the construct
includes sequences necessary for assemblying proteins to
15   package its corresponding RNA genome. The DNA construct
preferably excludes at least some of the viral sequences
that encode viral proteins, making it impossible for the
viral RNA genome to be packaged as an infectious virion
without the assistance of a helper viral vector. This
20   helps to assure that any retrovirion that is eventually
packaged with the assistance of a helper viral vector
will be capable of only an initial infection and will be
incapable by itself of inducing a spreading infection.
        Furthermore, removal of protein-encoding
25   segments very substantially reduces the total length of
retroviral sequences in the construct, allowing room
between the 5' and 3' LTR sequences for long foreign
sequences to be included in the construct while keeping
the recombinant retroviral sequences within the
30   construct to a length compatible with RNA genome
packaging. Retroviral vectors according to the present
invention may be packaged up to a length of about 9 kbp,
and by retaining only about 2-3 kbp of the viral
sequences, the construct can carry up to 6-7 kbp of
35   foreign genetic material within the region bounded by
the 5' and 3' insertion sequences. The extended length
of the foreign sequence that can be inserted represents

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an important advantage of retroviral vectors for gene
transfer applications.

The invention further provides recombinant
retroviral constructs from which all protein-encoding
5   sequences are deleted or truncated in a manner that
assures that no coat protein or coat protein fragments
are expressed within a cell that incorporates the viral
vector sequences. Complete impairment of the ability to
encode coat proteins eliminates the continuing
10   antigenicity of the packaged retrovirion derived from
the construct. This is important when the retrovirion
is used to directly infect an animal, as hereinafter
described. If initial infection by a recombinant
retrovirion does not induce antibody production,
15   subsequent infections by additional recombinant
retrovirion is permitted.

The various DNA fragments used to assemble the
DNA construct are cut and ligated by procedures that are
conventional in the art and will not be described in
20   detail herein. Proviral DNA is obtained, for example,
according to the method of Verma, I.M. et al., Proc.
Natl. Acad. Sci USA 77, 1773-1777 (1980) and Van Bevern
et al. Cell 27, 97-108 (1981).

The double-standard DNA construct generally
25   contains plasmid sequences because at each step of the
construction, multiple copies of the intermediate
recombinant DNA constructs are obtained by raising them
in plasmid form in a prokaryotic cell, such as E. Coli.
The amplifications of intermediate constructs also
30   represents purifications of the intermediate
constructs. The plasmid sequences that are outside of
the bracketed viral LTR sequences do not become part of
the retroviral genome and need not be considered for
genome packaging considerations; e.g., when considering
35   the length of an inserted segment.

A DNA segment that encodes a gene product is
selected according to the intended end utility of the

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retroviral vector. In some cases, the proteinaceous
product that is encoded by the selected DNA segment may
impart a selectable characteristic to the cell. For
example, described herein as an example of the present
5 invention is the introduction into cells of the human
hypoxanthine phophoribosyltransferase (hHPRT) gene by
retroviral infection. Genetic HPRT deficiency in humans
is associated with the devastating Lesch-Nyhan syndrome
and forms of gouty arthritis. An infectious retroviral
10 vector incorporating the hHPRT gene for the purpose of
incorporating the gene into cells may be used to
ameliorate the genetic deficiency. The HPRT gene
further imparts a selectable marker to the infected
cells, facilitating selection of cells that have
15 incorporated the gene.

        If the gene of interest is not readily
selectable, e.g., growth hormone (GH), it is generally
preferred that the retroviral vector include, in
addition to the gene of interest, a selectable marker
20 for purposes of amplification and identification.

        The DNA constructs are transfectable into
eukaryotic cells by standard technique, including the
well known calcium phosphate precipitation procedure,
Graham, F.L. et al., _Virology_ 52, 456-467 (1973). A
25 cell that is transfected with the construct may express
the products of the genetic sequences carried by the
construct. However, at this stage, gene product
expression is primarily important as a vehicle for
selecting cells that are transfected because many of the
30 advantages that accrue through the use of retroviral
vectors are not fully realized at this stage. Thus, the
transfected cell is generally considered only an
intermediate step in the method of the present invention.

        Recombinant retrovirions, from constructs that
35 are purposefully assembled so that they are not
self-packaging whereby the retrovirions may be used for
one-time infection of other cells, are obtained

("rescued") from the construct-transfected cells with
proteins expressed by "helper virus" vectors that are
also introduced into the transfected cells. The helper
viral vector encodes proteins that are not encoded by
5   the construct but which are necessary for packaging the
recombinant RNA viral genome corresponding to the DNA
construct. The helper viral vector is also of
retroviral derivation and is related to the parent
retrovirus from which the DNA construct was assembled,
10 at least to the extent that its packaging proteins will
package the recombinant retroviral genome.

As defined above, "retroviral vector" herein is
used to encompass several forms of retroviral genetic
material, and helper vector rescue may be accomplished
15 in a variety of manners, depending upon the requirements
of the particular application. A cell line may be
pre-altered, either by infection or transfection, so as
to incorporate a helper virus vector as DNA within its
genome, and thereby expressing helper viral coat
20 proteins. When a construct is subsequently transfected
into such a pre-altered cell line, the helper virus
vector-expressed coat proteins are used to package the
RNA genome of the construct. Alternatively, a construct
that contains genetic material of interest may be
25 transfected into a cell line and the cell line
subsequently infected with a helper retrovirion. As a
further alternative, helper viral constructs and
recombinant retroviral constructs may be cotransfected
into a cell line. In all cases, packaged recombinant
30 retrovirion is shed from cells that incorporate both the
construct and the helper virus vector.

The helper viral vector that is used in virion
rescue is preferably selected or constructed so as to be
innocuous in the end use of the recombinant infectious
35 retrovirion. For example, if the recombinant
retrovirion is to be used to carry genetic information
into somatic cells of a living animal, a helper virus

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may be selected which is known to cause no adverse
effects in the animal. Alternatively, and in many
instances preferably, the helper virus vector is itself
defective, lacking sequences needed to package its own
5   genome as an infectious virus. The helper viral vectors
are made defective by deleting their packaging sequences
while retaining their coat protein-encoding segments.
Cells transfected with a recombinant construct and also
incorporating a defective helper viral vector will shed
10   only recombinant retrovirions having genomes
corresponding to the construct and that contain the
desired genetic material. These retrovirions, though
directly infectable into cells for a one-time infection,
will not subsequently repackage their genome, and thus,
15   cannot by themselves create a spreading infection.

     The DNA constructs produced according to the
methods of the invention are further manipulatable to
incorporate additional genetic information. For
example, retroviruses which are initially constructed so
20   as to carry a gene that encodes a selectable protein may
be further altered to incorporate a gene that encodes a
nonselectable protein. This permits cells to which the
desired nonselectable gene has been imparted by the
retroviral vector to be selected through growth in an
25   appropriate medium from cells which have not
incorporated the genetic information carried by the
vector.

     Retroviral LTRs include strong promoter
sequences, and in many instances it is desirable to
30   insert a product-encoding DNA sequence into the
construct under the promotional control of the LTR
promoter sequences. However, it may be the case that
although a retroviral vector will incorporate its
genetic information into the genome of an infected cell,
35   the incorporated genetic information will not be
expressed to any significant extent due to various
cellular control mechanisms. In accordance with an

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important aspect of the present invention, it is found
that product-encoding DNA segments may be incorporated
into retroviral constructs linked to and under the
control of their own promoter or of another nonviral
5   promoter. A eukaryotic gene that is carried by a
retroviral vector into a eukaryotic cell under the
control of a nonviral promoter is found to express the
gene products irrespective of the functionality of the·
viral genetic information incorporated into the cell.
10 Self-promoted genes function independently of viral
regulatory elements, such as transcription promoters,
splice sites or polyadenylation signal. Thus, factors
which reduce or totally suppress viral gene expression,
which may be due to the failure of viral promoters or
15 other regulatory sequences to be activated in particular
cells, do not affect the expression of the gene promoted
by its own transcriptional regulatory signals. Evidence
that a retroviral vector can carry a protein-encoding
segment into a cell under control of its own promoter is
20 evidenced by the fact that encoding segments linked to
their own promoter express product irrespective of their
orientation (parallel or reversed) relative to the viral
LTR promoter sequences.

        Often, eukaryotic genes contain introns, i.e.,
25 noncoding segments. Because correct transcription of an
intron-containing gene requires regulatory mechanisms
present in the cell to which the gene is endogenous and
which may be absent in the host cell, it is generally
desirable to remove the introns prior to insertion of
30 the gene into a retroviral vector so that the gene will
be correctly expressed in whatever cell line the gene is
carried by the retroviral vector. Removal of introns
may be accomplished by production of cDNA by reverse
transcription from messenger RNA. cDNA contains only
35 protein-encoding sequences and therefore lacks upstream
promoter sequences. In those cases where it is
desirable to insert a eukaryotic gene into a retroviral

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vector under the promotional control of a nonviral
promoter, it is necessary to link a promoter to the cDNA
in appropriate fashion.

The promoter sequence may be the natural
5   promoter of the gene or may be another promoter selected
for particular properties. For example, a promoter that
is known to respond to external stimuli may be linked to
a cDNA segment, permitting expression of the retroviral
vector-inserted gene to be externally regulated after it
10   is incorporated into the genome of a cell. Examples of
externally regulatable promoter sequences include, but
are not limited to, promoter sequences for the following
genes: chicken transferrin gene, mouse metallothionein
I gene, mouse metallothionein II gene, mammalian
15   transferrin genes, mammalian histocompatibility genes,
mammalian ceruloplasmin genes, mammalian glycolytic
enzyme genes, mammalian albumin genes, mammalian
immunoglobulin genes, mammalian interferon genes, and
mammalian heat shock genes.
20   A further advancement of the present invention
is the novel use of amphotropic, replication-competent
helper viruses to rescue infectious retrovirions having
the construct genome. If rescued with amphotropic
helper viruses, the rescued virions are packaged in
25   proteins, particularly envelope (env) proteins, that
make them widely infective of cells of various species.
By amphotropic is meant the ability to infect cells of
relatively unrelated species, and herein the term
"amphotropic" shall be used to refer to retroviral
30   vectors which are infective across biological family
lines. Certain retroviral vectors are even sufficiently
amphotropic to cross phylum lines, e.g., between mammals
and birds. Amphotropism in a retroviral vector gives
the vector much wider utility, e.g., for imparting
35   genetic characteristics, such as rapid growth, to a wide
variety of domestic animals. It also allows a vector
that is intended for gene therapy in humans to be

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initially developed and grown in cell lines derived from a lower animal.

　　　　Among the important advantages afforded by retroviral vectors is the efficiency with which the genetic information of the retroviruses is incorporated into the genome of the cell. In certain retroviral infection protocols, substantially every cell is found to incorporate the retroviral genetic information. Another important advantage afforded by retroviral vectors is the general tendency to incorporate a single copy of the genetic material into the genome of each cell. If the cell to be infected is either a part of a living animal or is to be transplanted into a living animal, it is generally preferred that the cell contain a single copy of the genetic material.

　　　　An important use of retroviral vectors is for gene therapy in vertebrate animals, including fishes, birds and mammals. Retroviral vectors are expected to be useful in correcting genetic deficiencies in humans. As one method of performing gene therapy on an animal, a cell line is infected with recombinant retrovirion, and infected cells that are found to express the product(s) of a gene(s) carried into the cells by the retrovirion are transplanted into the animal. It is demonstrated that such genetically altered cells continue to express gene product within the recipient animal. Preferably, the cell line that is selected for use in this type of gene therapy is rapidly proliferating, e.g., the systemic stem cells of the bone marrow.

　　　　The invention will now be described in greater detail by way of specific examples.

<u>EXAMPLE 1</u>

　　　　Assembly of two constructs containing retroviral sequences and an hHPRT-encoding sequence is diagrammatically outlined in FIGURE 1. The recombinant plasmids were constructed using standard techniques as described in Maniatis, T. et al., <u>Molecular Cloning: A</u>

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Laboratory Manual (Cold Spring Harbor Laboratory, Cold
Spring Harbor, N.Y.) and Verma et al. (1980) supra. In
Figure 1, abbreviations of common restriction enzymes
are P, Pst I; B, Bam HI; H, Hind III; E, Eco RI; R. Rsa
5   I; Hp, Hpa I; S. Sal I. To reduce unwanted ligation
products, the indicated fragments were treated with
bovine alkaline phosphatase (BAP). Cross-hatched
segments represent hHPRT cDNA, boxes with interior
arrows represent viral LTRs with orientation indicated
10   by the arrow (5' -- 3'), thin lines indicate viral
sequences, thick lines indicate plasmid pBR322
sequences, and wavy lines indicate mouse cell sequences
surrounding proviral DNA.

      The cDNA clone of the gene for hHPRT was
15   obtained from the SV40-based expression vector p4aA8 in
which it was initially isolated, Jolly, D.J., et al.,
Proc. Natl. Acad. Sci. USA 80, 477-481 (1983). Viral
LTRs were linked to the hHPRT cDNA such that the coding
regions for viral proteins were either completely
20   removed (gag and pol) or truncated (env); thus, no viral
proteins would be expressed from the contructs. The 5'
LTR and adjacent sequences were derived from a cloned
Mo-MSV provirus, pMSV$_1$ IL, Van Beveren et al., Cell
27, 97-108 (1981), and the 3' LTR and adjacent sequences
25   were obtained from cloned unintegrated Mo-MuLV DNA,
pMLV-1, Berns et al., J. Virol; 36 254-263, (1980);
Shinnick et al., Nature (London), 293, 543-548 (1981).
Retained in the constructs are 5' sequences presumably
involved in packaging of viral RNA, Shank, P.R. J. Virol
30   36, 450-456 (1980). The hHPRT gene is situated so that
the ATG start codon is in approximately the same
position as the start codon for the gag polyprotein in
the parental virus.

      Two potential polyadenylylation (poly A)
35   signals, which are expected to terminate transcription
and thereby reduce the efficiency of the construct,
exist near the 3' end of the Pst I to Bam HI hHPRT cDNA

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fragment. Thus, in addition to the pLPAL construct
containing these sites, another construct, pLPL, was
made in which these sites were removed and replaced with
Mo-MuLV sequences. In pLPL, the hHPRT cDNA was joined
5   to the viral env gene downstream of the env splice
acceptor junction and start codon. Thus, pLPL should be
transcribed as a single RNA species of about 4.0 kb,
which would encode hHPRT.

          Cell lines and tissue cultures used in this
10   Example are as follows. The 208F hHPRT⁻ clonal cell
line was derived from Fischer rat cells by selection in
6-thioguanine, Quade, K., _Virology_ 98, 461-465 (1979).
The BALB 3T3 hHPRT⁻ cell line 2TGOR, Jha, K.K. et al.,
Somat. Cell Genet. 6, 603-614 (1980), was a gift of H.
15   L. Ozer (Hunter College, New York). The hHPRT⁺ rat
cell line WA, derived from Wag-Rij rats, was a gift from
M. Maszynsky (Case Western University) and G. Rosenfeld
(University of California at San Diego). All cell
lines, including NIH 3T3 TK⁻ cells, Wei, C. et al.,
20   _J. Virol._ 39, 935-944 (1981); SV40-transformed hHPRT⁻
Lesch-Nyhan (LNSV) cells, Bakay, B. et al., _J. Cell Sci._
17, 567-578 (1975); GM637 SV40-transformed normal
(hHPRT⁺) human fibroblasts, Tan, Y. H. et al. _J. Gen._
Virol. 34, 401-411 (1977); and D98 hHPRT⁻ HeLa cells,
25   Francke, U., _Chromosoma_ 41, 111-121 (1973) were
maintained in Dulbecco-Vogt modified Eagle's medium, 10%
fetal bovine serum (Sterile Systems, Logan, UT).
hHPRT⁺ cells were selected in the same medium
containing 30 μM hypoxanthine/1.0 μM amethopterin/20 μM
30   thymidine (HAT medium).

          For transfecting cells with the constructs,
hHPRT⁻ cells were plated at 5 X 10⁵ cells per 5-cm
dish on day one. On day two, 0.5 μg of uncut
recombinant plasmid DNA and 8 μg carrier DNA (from NIH
35   3T3 TK⁻ cells) were transfected into the cells using
the calcium phosphate precipitation procedure of Graham,
F.L. _Virology_ 52, 456-467 (1973); Corsaro, C.M. et al.,

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<u>Somat-Cell Genet.</u> <u>7</u>, 603-616 (1981). On day three, the
cells were split 1:5 into HAT medium, and every three
days thereafter, the medium was replaced with fresh HAT
medium. For quantitation, colonies were stained on day
5   nine with Coomassie blue (1 g/liter in 50% methanol/10%
glacial acetic acid/40% water). In a transfection
assay, recombinant plasmids, pLPA, pLPAL, and pLPL,
conferred HAT resistance (HAT^r) to HAT-sensitive
(HAT^s) hHPRT$^-$ 208F rat cells. The efficiency of
10 HAT^r colony production was the same for the constructs
as for the original SV40-hHPRT hybrid plasmid p4aA8 in
which hHPRT is expressed from a SV40 promoter (400-700
colonies per $\mu g \cdot 10^6$ cells). No HAT^r colonies were
observed in control 208F cells.

15   Cells transfected with the hHPRT-containing
viral constructs are unable to produce virus because the
coding regions for the viral proteins that are necessary
for viral replication have been deleted. However,
defective viral genomes were rescued with
20 replication-competent helper virus. hHPRT-expressing
virus was rescued from cells using replication-competent
helper virus, either Moloney murine leukemia virus
(Mo-MuLV), Fan, H. et al., <u>J. Virol.</u>, <u>14</u> 421-429 (1974),
FBJ murine leukemia virus (FBJ-MuLV), Curran, T. et al.,
25 <u>Virology</u> <u>116</u>, 221-235 (1982), or 1504A amphotropic virus
Rasheed, S. et al., <u>J. Virol.</u> <u>19</u>, 13-18 (1976). The
later amphotropic virus is widely infective in a variety
of vertebrate cells, having an envelope protein that
enables it to infect, for example, chicken, rodent as
30 well as human cells.

          Medium from helper virus-producing cells was
filtered through a 0.45 μm filter and applied to cells
at a multiplicity of about 1 in the presence of
Polybrene (Sigma) at 4 μg/ml. The cells were passaged 3
35 days after infection, medium was replaced with fresh
medium on day five, and supernatant was harvested on day
six from nearly confluent cells. The supernatant was

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centrifuged at 5,000 x g for 7 min. to remove cells, and
aliquots of the supernatant were applied to 208F
hHPRT⁻ and NIH 3T3 TK⁻ cells (plated the day before
at 5 x 10^5 per 5-cm dish) in medium containing

5   Polybrene at 4 μg/ml. hHPRT⁺ 208F cells were scored
as in the transfection assay, and Mo-MuLV and FBJ-MuLV
were quantitated by using the XC plaque assay of Rowe,
W.P. et al., _Virology_ 42, 1136-1139, on NIH 3T3 TK⁻
cells. 1504A amphotropic virus is XC-plaque negative

10   and was not quantitated.

In initial experiments, hHPRT⁻ rat cells
transfected with pLPAL or pLPL were infected with helper
virus and the virion produced by these cells was able to
confer HAT resistance to hHPRT⁻ cells. Furthermore,

15   transmissible hHPRT-virus was also obtained when
molecularly cloned helper virus DNA was cotransfected
with pLPAL or pLPL. Under both of these conditions, the
relative titer of hHPRT-expressing virus was uniformly
low (1% of helper virus titer). Analysis of the

20   transfected cells revealed a number of aberrant forms of
viral RNA, which may not be properly packaged or may
produce noninfectious virions.

In an attempt to obtain high hHPRT-expressing
virus titers, the following strategy was used. hHPRT⁻

25   rat cells were infected with hHPRT-expressing virion
obtained from helper virus-infected pLPAL- or
pLPL-transfected rat cells. HAT^r clones that did not
produce helper virus (nonproducer cells) were isolated.
Nonproducer cell lines were superinfected with helper

30   virus and the culture medium was assayed for

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hHPRT-expressing virus and helper virus. As shown in
Table 1 below:

TABLE 1

5 Rescue of HPRT-expressing virus*

Cells infected	Mo-MuLV		FBJ-MuLV		1504A	
	cfu/ml	pfu/ml	cfu/ml	pfu/ml	cfu/ml	pfu/ml
208F	<10	$10^{4.4}$	<10	$10^{3.8}$	<10	<10
208F-LPAL	$10^{1.7}$	$10^{4.6}$	$10^{2.0}$	$10^{3.9}$	$10^{1.7}$	<10
208F-LPL	$10^{3.3}$	$10^{4.8}$	$10^{3.0}$	$10^{4.1}$	$10^{3.5}$	<10
2TGOR-LPL	$10^{5.3}$	$10^{6.3}$				

*hHPRT-expressing virus was rescued from cells by using
various helper viruses. Results are expressed as
hHPRT$^+$ colony-forming units (cfu) and helper virus XC
15 plaque-forming units (pfu). The amphotropic virus 1504A
does not form plaques in the XC assay and was not
quantitated.

Mo-MuLV, FBJ-MuLV, and the amphotropic virus were able
to rescue hHPRT-expressing virus. Although
hHPRT-expressing virus could be rescued from cells
20 infected with either LPAL or LPL, the titer from cells
infected with the LPAL virion that contains the hHPRT
polyadenylylation signals was no more than 10% of that
cells infected with LPL from which these signals had
25 been deleted. This result confirms the hypothesized
advantage of removing poly A sites from retroviral
constructs. Averaged over many experiments, the
hHPRT-expressing virus/helper virus ratio using the
LPL-infected nonproducer line was about 10%.
30   The relatively low titers of hHPRT-expressing
virus rescued from 208F rat cells refects the
inefficient replication of murine retroviruses in rat
cells. In another experiment, a hHPRT$^-$ murine cell
line, 2TGOR, was infected with virion from rat 208F
35 cells producing hHPRT-expressing virion (LPL) and
Mo-MuLV. HAT^r cells were selected and passaged once
to allow for helper virus spread. Supernatant from a

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nearly confluent dish of these hHPRT$^+$ mouse cells had
a titer of 2 x 10^5 hHPRT-expressing virus and 2 x
10^6 of the Mo-MuLV helper virus (Table 1). Thus, high
titers of hHPRT-expressing virus can be generated in an
5   appropriate cell line.

One important aspect of the present invention
is the selection in certain applications of an
amphotropic helper virus that rescues recombinant
viruses in a manner that the rescued virions are
10   generally infective in vertebrate cells, and
-particularly those that are infective of human cells.
If a viral genome is packaged with proteins encoded by
an amphotropic helper virus vector, either natural or
recombinant, even though the retrovirus from which the
15   recombinant genome is derived is specifically infectious
of a single species or limited number of related
species, the packaged recombinant retrovirion is able to
infect a variety of cell types from a variety of
species. This is because it is the packaging proteins,
20   rather than the RNA genome, that determines which cells
the recombinant virus is able to infect. Accordingly,
one of the helper virions selected for these experiments
was the above-mentioned amphotropic helper virus 1504A
which is able to infect human cells.

25   All hHPRT$^-$ human cells tested, including D98
HeLa cells, SV40-transformed Lesch Nyhan (LNSV)
fibroblasts, and Epstein-Barr virus-transformed
Lesch-Nyhan lymphoblasts, could be converted from HAT^s
to HAT^r by infection with culture medium from
30   LPL-infected nonproducer rat cells that were
superinfected with amphotropic virus. The efficiency of
formation of HAT^r colonies was similar to that
obtained for infection of 208F rat cells.

The hHPRT activity of the hHPRT-expressing
35   virus-infected LNSV cells was analyzed to ascertain
whether authentic human hHPRT protein was present and at
what levels. Several rat cell lines that had been

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transfected or infected with the hHPRT-expressing viral
vectors were also analyzed. Both the LNSV cells and rat
208F cells infected with hHPRT-expressing virus express
hHPRT protein that comigrates on polyacrylamide gels
5   with authentic human hHPRT from HeLa or GM367 cells and
migrates differently from rat hHPRT from WA cells.
Control LNSV cells and 208F hHPRT$^-$ rat cells have
undetectable activity in this assay. The levels of
enzyme activity in LNSV and rat cells infected with
10   hHPRT-expressing virus obtained from the LPL construct
are similar to those of control hHPRT$^+$ human cells.
208F rat cells infected with hHPRT-expressing virus from
the LPAL construct display human hHPRT activity but at a
significantly lower level than the LPL-infected cells.
15   The RNA from cells either transfected or
infected with the hHPRT retroviral vectors was analyzed
to determine whether the RNA patterns corresponded to
those predicted from the DNA organization of the
vectors. Two probes were used, one to hHPRT cDNA and
20   the other to the Mo-MSV LTR. The LTR probe does not
hybridize to RNA transcripts from control 208F cells
while the hHPRT probe hybridizes slightly to control
cell RNA. A complex pattern of RNA transcripts
hybridizing to either the LTR or hHPRT probe was
25   observed in cells transfected with the vectors. RNA
blot analysis of RNA from cells infected with hHPRT
virus, on the other hand, produced a readily
interpretable pattern because viral integration is
precise with respect to the viral genome. In cells
30   infected with hHPRT-expressing virus from
pLPAL-LPAL-transfected cells, two species of RNA
hybridizing to both probes were identified. The size of
the larger RNA species corresponds to the full-length
RNA transcript expected from LPAL (3.9 kb). The smaller
35   species of RNA (1.6-1.9 kb) probably represents
transcripts that initiated at the 5' LTR but terminated
at the putative polyadenylylation signals in the hHPRT

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cDNA fragment. In hHPRT(LPL)-expressing, virus-infected
cells, only a single species of RNA hybridized to either
the hHPRT probe or the LTR probe. The size of this RNA
corresponds to the full length RNA (4.0 kb) expected
5   from LPL if the normal retroviral transcriptional
signals are used. The fact that all major species of
RNA in hHPRT-expressing virus-infected cells hybridize
to both viral LTR and hHPRT probes is consistent with
the idea that transcripts initiate in the LTR and
10   transcribe through the hHPRT cDNA sequences.

To determine the organization of chimeric
proviral DNA in hHPRT-expressing virus-infected cells,
the DNA in recipient LNSV cells was analyzed by using
restriction endonucleases. The restriction endonuclease
15   Sst I cleaves only once in each LTR of pLPL and does not
cut in the SV40-based expression vector p4aA8. When
high molecular weight DNA from LNSV cells was cleaved
with Sst I and then hybridized to a hHPRT probe, two
fragments of about 14.5 and 7.2 kbp were identified.
20   These fragments presumably represent the endogenous
hHPRT gene of LNSV cells. In cells transfected with the
hHPRT expression vector p4aA8, an additional fragment
larger than 25 kbp was detected, which is consistent
with integration of the p4aA8 plasmid in a tandemly
25   repeated unit, Perucho et al., Cell 22, 309-317 (1980),
which would not have internal Sst I sites. Sst
I-cleaved DNA from LNSV cells that were either
transfected or infected with LPL vectors shows the
control cell DNA fragments plus a single new fragment of
30   about 3.9 kbp, as expected. Thus, like other
retroviruses, the hHPRT-proviral DNA appears to have an
uninterrupted 5' LTR-hHPRT-LTR 3' organization.

The size of the hHPRT-expressing virus is
small, less then 4.5 kb, including both LTRs. It should
35   be noted that only the construct segments from the 5'
LTR through 3' LTR comprises the viral genome, the
surrounding plasmid sequences that are used for

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construct replication and selection comprising no
portion of the genome that is transcribed from the
construct and subsequently packaged. At the 5' end of
the virus, 1.8 kb includes the 5' LTR, the tRNA binding

5   site, the presumed packaging signal, and the hHPRT
cDNA. About 0.7 kb at the 3' end includes the 3' LTR
and sequences involved in the synthesis of plus-strand
DNA during viral infection. Thus, only 2.5 kb of the
viral sequences are required for hHPRT expression and

10 virus rescue. Therefore, 6-7 kb of additional sequences
could be added to the hHPRT-encoding virus without
exceeding the limit for retroviral genome size. Hence,
this vector can be used as a vehicle for other genes and
carries a convenient and powerful selectable marker.

15   EXAMPLE 2
The helper viruses used in Example 1 are
themselves infectious, being able to package their own
genome and be shed as packaged viruses from infected
cells. For certain applications, particularly for

20 eventual end use for gene therapy in humans, it is
undesirable to have infectious helper viruses which may
have the potential to induce tumors. It may be noted,
however, that many replication-competent viruses, like
FBJ MULV virus, T. Curran, N. Teich, Virology 116,

25 221-235 (1982), have never been shown to cause any
disease. Nevertheless, to avoid any likelihood of a
helper virus inducing a tumor or other pathogenic
condition, the invention provides defective helper
viruses which cannot package their genomes but which

30 encode proteins needed to package the recombinant
retroviral vector. Construction of a defective helper
virus pSEM is diagrammed in FIG 2. pMLV-intermediate is
described by Berns et al., J. Virol. 36, pp. 254-263
(1980). pMLV-48 (MO-MLV provirus) is described by

35 Bacheler et al., J. Virol. 37, p. 181-190 (1981)
Briefly, molecularly cloned MO-MLV DNA
containing the coding region for gag-pol-env and 3'LTR

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sequences are linked to a DNA fragment containing SV40
promoter and enhancer elements as well as splice
donor-acceptor sites for viral coat protein. Thus the
5' LTR insertion and promoter sequences as well as the
5   closely adjacent viral packaging sequences are replaced
by SV40 insertion and promoter sequences with no
adjacent packaging sequences. Replacement of the 5' LTR
insertion segments with non retroviral sequences having
promoter sequences eliminates the retroviral
10   recombinations that frequently occur when both 5' and 3'
LTR segments are present.

Rescue of a recombinant construct, such as pLPL,
with a defective helper vector, e.g., by cotransfection,
generates infectious LPL virion without generating any
15   helper virus upon subsequent infection of recipient
cells. A variety of sensitive assays designed to
determine infectious helper virus particles gave
negative results. It is estimated that titers of 10^4
LPL virus are released from cells cotransfected with
20   pSEM. The titers can be increased if appropriate
indicator cells are used for hHPRT assays.

EXAMPLE 3

The defective helper virus construct, pSEM,
described above in Example 2, contains env sequences
25   that limits its infectivity to rodent cells. It is, of
course, desirable to be able to infect other types of
cells, particularly human cells, with the recombinant
virions, and in accordance with an important aspect of
the present invention, amphotropic defective helper
30   viruses are provided that are generally infective into
mammalian cells, and particularly, that are infective
into human cells. Construction of the DNA construct,
pSAM, of such a helper virus from pSEM is
diagrammatically illustrated in FIGURE 3. The construct
35   utilizes segments of amphotropic murine retroviruses
that are excised from 4070A clone (Chattopadhygy et al.,
J. Virol. 39, 779-791 (1982) and that have been shown to

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infect not only rodent cell but cells of a large array
of other species, including human cells. The most
significant difference between pSAM and pSEM is the
modification of pSEM to contain the env gene from the
5   molecularly cloned amphotropic viral DNA. Thus, viruses
that are rescued upon cotransfection with pSAM are able
to infect both rodent and nonrodent cells, including
human cells. Titers as high as 10^7 hHPRT units per
ml. of virus rescued with pSAM vector have been obtained.
10   EXAMPLE 4
        Mouse bone marrow cells were co-cultivated with
rat cells A.D. Miller, et al., Proc. Natl. Acad. Sci.
USA 80, 4709 (1983), producing HPRT-virus and helper
virus (Moloney strain; MoMLV (H. Fan, J. Virol. 14, 421
15   (1974))), and then transplanted into syngeneic mice.
Bone marrow cells were selected for infection because 1)
it is likely that pluripotent self-renewing hematopoetic
stem cells present in bone marrow are among those cells
which can be infected R.J. Eckner, et al., J. Exp. Med.
20   149, 340 (1979), ii) it has been demonstrated that
isologous bone marrow chimeras are highly sensitive to
chronic MoMLV R.J. Eckner, et al., Virol. 122, 171
(1982), and iii) bone marrow cells are readily
accessible and have a high proliferation rate after
25   reintroduction into mice. To maximize the possibility
of infection, the bone marrow cells were overlayed onto
monolayers of virus-producing adherent cells for two
days. The infected cells were then injected into
lethally irradiated mice of the same strain as the donor
30   mouse. The dose of radiation was chosen so that
transplantation of fresh bone marrow was a requirement
for survival of the mice. Thus certain hematopoetic
cellular compartments (bone marrow and spleen) depleted
by irradiation are repopulated by the virus-infected
35   bone marrow cells.
        Bone marrow cells from the spine of a C57BL/6
mouse were removed R.J. Eckner, et al., Virol. 122, 171

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(1982), and aliquots of 5×10^7 nucleated cells were
overlayed into 10 cm dishes of confluent 208F rat cells
that produce HPRT$^+$-virus and MoMLV helper virus A.D.
Miller, et al., _Proc. Natl. Acad. Sci. USA_ 80, 4709

5   (1983). The rat cells produce virus such that medium
harvested from these cells after 16 hr contains about 5
$\times 10^3$ HPRT$^+$ cfu/ml with a ten-fold excess of helper
virus. Viral infections were performed in Dulbecco-Vogt
modified Eagle's medium containing 8 μg/ml Polybrene

10   (Sigma Chemical Co.) and 10^{-5} M 2-mercaptoethanol.
After 16 h, non-adherent cells were removed, resuspended
at 2×10^7 viable cells/ml (85% of the cells were
viable as judged by trypan blue dye exclusion), and 0.5
ml aliquots were injected intravenously into lethally

15   irradiated (1200 R from a ^{60}Co source) C57BL/6
recipient mice. Irradiated control mice not receiving
bone marrow all died of hematopoetic failure within 15
days after irradiation.

        Two months after the bone marrow transplant,

20   protein from spleen was analyzed electrophoretically for
the presence of human HPRT. In the spleen from the
surviving mouse receiving infected bone marrow, a form
of HPRT which comigrates with one human HPRT isoenzyme
is clearly visible. On longer exposures, a band

25   comigrating with the other human isoenzymes can also be
detected.

        These results indicate that the human HPRT gene
can be expressed in transplanted mouse bone marrow cells
following co-cultivation with HPRT-virus producing cells.

30   EXAMPLE 5

        A technical problem with the experiment
described in Example 4 was the low survival rate
(10-20%) of the irradiated recipient mice. Poor
survival appeared to be due to loss of viability of bone

35   marrow stem cells able to rescue recipient mice from
hematopoetic failure after long term co-cultivation, as
bone marrow maintained for 3 hours _in vitro_ and used for

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transplantation resulted in nearly 100% survival. To
infect a high proportion of cells in this relatively
short time period, a higher titer virus stock than was
available from the existing virus-producing line was
5   required.

To generate a cell line producing a high titer
of HPRT-virus, construct pLPL and the packaging vector
pSAM were cotransfected, F.L. Graham, A.J. van der Eb,
Virology 52, 456 (1973); M. Wigler, et al., Cell 14, 725
10   (1978); C.M Corsaro, M.L. Pearson, Somatic Cell Genetics
7, 603 (1981), into HPRT$^-$ Balb/3T3 cells, and HPRT$^+$
clones producing high titer virus capable of converting
HPRT$^-$ mouse or human cells to HPRT$^+$ were selected.
(Ouabain resistant HPRT$^-$ (6-thioguanine resistant)
15   Balb/3T3 cells (B77/OTG) were a gift from Dr. Harvey
Ozer and were transformed with Rous sarcoma virus and
then selected for resistance to ouabain and
6-thioguanine.) Culture medium exposed to a confluent
monolayer of cells derived from one clone (c7cl) for 12
20   h contains about 10^7 HPRT-virus per ml, when assayed
on HPRT$^-$ rat cells, HPRT$^-$ human (LNSV cells) or
HPRT$^-$ Balb/3T3 cells as indicator cells. Because the
virus released from c7cl cells is capable of infecting
human or mouse cells, it has the properties of an
25   amphotropic virus. Naturally occurring amphotropic
viruses, including the virus generated from the
molecular clone used in the construction of pSAM, are
N-tropic, i.e., have reduced ability to infect cells of
Balb/c mouse origin. However, the virus produced by
30   c7cl can infect Balb/3T3 cells with high efficiency, as
expected N. Teich, in RNA Tumor Viruses, R. Weiss, et.
al., eds. (Cold Spring Harbor Laboratory, New York) Ch.
2, pp209-260 (1982), due to the presence of the
NB-tropic p30gag region from Mo-MLV in pSAM.
35   The HPRT-virus produced by c7cl had high titer
and a wide host range. Virus produced by c7cl contains
less than 0.1% of helper virus, an insignificant titer.

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The high titer amphotropic HPRT-virus obtained
from clone c7cl was used to infect mouse bone marrow
cells. Mouse bone marrow cells were infected using
HPRT-virus harvested from a clonal Balb/3T3 cell line
5   (c7cl) cotransfected with the HPRT expression vector
pLPL and the packaging vector pSAM. The titer of the
virus stock used was 2×10^7 HPRT-virus/ml. Bone
marrow cells obtained from mouse spine were exposed to
HPRT-virus in two one-hour incubations at room
10 temperature. 100 ml virus stock was mixed with $5 \times
10^8$ bone marrow cells. After each incubation, the
cells were centrifuged at 600 g for 10 min and the
medium removed. Prior to injection, the cells were
suspended in 10 ml medium containing 10^7 MoMLV helper
15 virus. 0.5 ml doses of suspended bone marrow cells were
injected into the tail veins of mice previously exposed
to 1200 R from ^{60}Co radiation source.

After 3 months, spleen and bone marrow (spine)
cells were collected and overlayed on recipient cells
20 for virus assay. HPRT-virus assay was performed by
overlay of 10^7 mouse cells on 5×10^5 HPRT$^-$
ouabain resistance (oua^r) Balb/3T3 cells in a 5 cm
dish. After 16 h, the dishes were washed 3 times to
remove loosely adherent mouse cells, trypsinized, and
25 split 1:5 into medium containing 30 μM hypoxathine, 1 μM
amethopterin, 20 μM thymidine (HAT medium) and 5 mM
ouabain.

Colonies were counted after 6-10 days. MoMLV
helper virus was quantitated using the XC plaque assay,
30 W.P. Rowe, W.E. Pugh, J.W. Hartley, Virology 42, 1136
(1970). Briefly, 10^7 mouse cells were overlayed on 5
$\times 10^5$ NIH 3T3 TK$^-$ cells for 16 h in a 5 cm dish.
The dishes were washed 3 times and split 1:10. Three
days later the cells were UV irradiated and overlayed
35 with XC cells. Two days later, the cells were stained
and placques were counted. The amphototropic virus is
XC$^-$ and does not score in this assay. Amphotropic

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virus was quantitated using the S^+L^- assay, R.J.
Eckner, K.L. Hettrick, _J. Virol._ _24_, 383 (1977). 10^7
mouse cells were overlayed on 10^5 CCC-81 cat cells in
a 5 cm dish. After 16 h, the dish was washed and the

5   cells were split 1:10 into dishes containing 2 x 10^5
rat kidney (NRK) cells. After 5 days, transformed foci
resulting from the helper virus-mediated transfer of the
sarcoma virus present in the cat cells into the NRK
indicator cells were counted. MoMLV does not score in

10   this assay because of its inability to infect cat cells,
and thus rescue the replication defective transforming
virus harbored by these cells. Titers of MoMLV were
approximately 10^6 XC plaque forming units per 10^7
cells per 16 h and the amphotropic virus ranged from

15   10^3 - 7 x 10^3 focus forming units per 10^7 cells
per 16 h. This shows that a chronic infection is
induced in the recipient mice.

          The survival rate of these mice (no deaths in
20 mice receiving transplants) was much higher than than

20   of the mice receiving bone marrow after 12-24 h
cocultivation with virus producing cells. Irradiated
mice that did not receive bone marrow transplants died
within 10 days. Spleen and bone marrow cells from some
of the mice receiving infected bone marrow were analyzed

25   for production of HPRT-virus. One expects that some
hematopoietic cells from the mice receiving infected
bone marrow should be infected with both the HPRT-virus
and helper virus, and thus should be producing
HPRT-virus. Spleen and bone marrow cells from mice

30   receiving bone marrow transplants were removed and
overlayed onto adherent HPRT$^-$ ouabain resistant
(oua^r) cultured mouse cells. One day later, the
dishes were washed thoroughly to remove unattached
cells, and the cells were exposed to selective medium

35   containing HAT and ouabain. This selection ensures that
only cultured cells converted to HPRT$^+$ by virus will
survive, as ouabain kills the donor mouse cells, and HAT

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selects against HPRT⁻ cells. The development of
colonies of cells surviving HAT selection are thus
dependent on HPRT-virus production by the donor cells.
HPRT-virus continued to be produced by spleen and bone
5   marrow cells of mice infected with the high titer
HPRT-virus even after 133 days following
transplantation, and demonstrates chronic persistence of
the HPRT-virus in these mice.
        DNA from cultured cells converted to HPRT⁺
10   by exposure to spleen or bone marrow from mice receiving
bone marrow transplants was analyzed for the presence of
HPRT-viral DNA to determine if the HPRT-virus was indeed
responsible for colony formation and that no
rearrangement of the virus had occurred. Cultured cells
15   converted to HPRT⁺ by the same virus stock used to
infect mouse bone marrow was also analyzed. Cellular
DNA was digested with SstI cuts in both LTRs of the
virus; thus a single band of about 3.9 kb was expected
to be present in all infected cells, and the
20   HPRT-specific radiolabeled probe hybridized to a DNA
species of about 3.9 kb from cells infected with stock
virus from c7cl cells. However, there was no
hybridization to a similar size DNA in uninfected
cells. An additional band of about 9 kb representing
25   the endogenous mouse HPRT DNA could also be seen in both
samples. The 3.9 kb band representing the HPRT-viral
DNA is present in cells converted to HPRT⁺ by exposure
to cells from infected mice. Thus viral DNA is present
in colonies of HPRT⁺ cells produced by exposure to
30   cells from infected mice, and the virus has the same
organization and is apparently identical to that
originally used for infection of the mice.
        Results presented here show that retroviral
vectors can be used to transfer genetic material into
35   intact animals. Two types of observations justify this
claim: i) bone marrow and spleen cells of mice
receiving infected bone marrow became chronic producers

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of HPRT-virus, receiving infected bone marrow became
chronic producers of HPRT-virus, additionally, the
released virus appeared to be identical to the virus
used for infection of the engrafted bone marrow, and ii)
5   human HPRT was expressed in the spleen of a recipient
mouse.

This experiment further demonstrates that gene
therapy can be performed by using a recombinant
retrovirion to carry a gene into a transplantable cell
10 line and then transplanting the cell line into a living
animal.

Lesch-Nyhan disease is a devastating human
disease which is apparently caused by a defect in the
HPRT gene W.N. Kelley, J.B. Wyngaarden, in The Metabolic
15 Basis of Inherited Disease, 5th edition (ed. J.B.
Stanbury et al.) McGraw Hill (1983); C.T. Caskey, G.D.
Kruh, Cell 16, 1 (1979).   One of the long term goals of
HPRT-research is to test the utility of retroviral
vectors as potential agents for correction of this
20 defect in somatic cells.   It has recently shown that
lymphoblasts from Lesch-Nyhan patients can be infected
with the HPRT-virus in vitro R.C. Willis, et al.,
J. Biol. Chem., 259, 7892 (1984).   The utility of
retroviral vectors for somatic cell modification may
25 require the availability of cell lines which provide
high titers of retrovirus containing foreign genes which
are capable of infection but not further propagation.
The amphotropic packaging construct described here only
partially meets these criteria. However, there has not
30 been observed any disease or gross pathology in infected
mice.   In addition, the HPRT-virus was designed so that
no viral proteins are synthesized, thus minimizing the
possibility of antigenic rejection of HPRT-virus-infected
cells.
35   Although in this experiment, the gene carrying
cells were introduced in replacement of substantially
destroyed bone marrow tissue, techniques are available

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for enhancing the relative proportion of genetically
altered transplanted somatic cells relative to the
endogenous population of cells. For example, a
retroviral vector may carry, in addition to a gene of

5   interest, a dihydrofolate reductase (dhfr) gene. If
bone marrow cells infected with the retrovirion are
introduced into the animal and then the animal is
administered controlled doses of methotrexate, the
transplanted, gene-carrying bone marrow cells should

10 exhibit a selective advantage relative to the endogenous
bone marrow cells.

EXAMPLE 6

In this example, the construct pLPL, described
above in Example 1, was further modified to incorporate

15 a rat growth hormone "mini-gene" under its own
promoter. Construction of the mini-gene is illustrated
in FIGURE 4A, and its insertion into the pLPL construct
is illustrated in FIGURE 5A. Briefly, the mini-gene
(FIG. 4B) consists of rat growth hormone cDNA produced

20 from rat growth hormone messenger RNA by reverse
transcriptase. To the cDNA is linked the normal rat
growth hormone promoter sequence. The cDNA contains no
intervening sequences (introns) which might prevent the
gene from being correctly transcribed in foreign cells

25 into which it is introduced. The pLPL construct is cut
at the Hpa I restriction site, and at this site, the rGH
mini-gene obtained from a growth mini-gene carrying
plasmic (pGH) by cleavage with Hind III is inserted by
blunt end ligation after filling in the ends using the

30 Klenow fragment. By this procedure, it is expected that
some of the mini-gene will be inserted in parallel with
the viral LTR promoter sequences and that some of the
mini-gene will be inserted in reverse orientation with
respect to the viral LTR promoter sequences. The viral

35 genome corresponding to parallel insertion is shown in
FIGURE 5b.

DNA constructs representing each orientation of

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the minigene, LPGHL (parallel), and LPHGL reverse, were
introduced (10 μg/5 cm dish of cells) into 208F HPRT⁻
rat cells by calcium phosphate precipitation (exp. 1).
One day after transfection, the cells were trypsinized

5   and seeded into HAT medium. HPRT⁺ colonies were
visible in about 5 days, and were pooled for analysis.
For assay of GH production, the cells were seeded at
about 20% confluency in growth medium containing 10%
fetal bovine serum with no added inducers (control) or

10   with 10^{-6} M dexamethasone (dex) or 10^{-6} M dex and
10^{-7} T_3. After 3 days, GH in the medium was
measured using an NAIMDD radioimmunoassay kit and rat GH
standard NIAMDD-rGH-I-4, Doehmer, J. et al., Proc. Natn.
Acad. Sci USA, 79: 2268-2272 (1982). Results are

15   expressed in Table 2 in ng GH per ml of medium.

Fold induction was calculated as the ratio of
GH production with dex + T_3 compared to control (GH
production from uninduced cells). Fetal bovine serum
contains low levels of inducers of GH production, thus

20   the fold induction may be underestimated.

In a separate experiment (exp. 2), the
transfected cells were infected with MoMLV helper virus
(MLV-K, ref. 31) passaged twice, and virus production
was measured in medium harvested under 16 h from a

25   confluent dish of cells. HPRT⁺ colony forming units
per ml of medium (cfu/ml) were measured on 208F rat
cells and MoMLV plaque forming units per ml (pfu/ml) on
NIH 3T3 TK⁻ cells.

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TABLE 2

ANALYSIS OF TRANSFECTED CELLS FOR VIRUS RESCUE AND GROWTH HORMONE PRODUCTION

| Transfected DNA | Growth Hormone Production | | | | Virus Rescue | |
	Control	dex	dex+T$_3$	Fold-Induction	HPRT$^+$ cfu/ml	MoMLV pfu/ml
pLPGHL						
Exp. 1	11	23	24	2.2	6.5×10^2	8×10^4
Exp. 2	22	102	99	4.5	3.2×10^2	8×10^4
pLPGHL						
Exp. 1	29	83	101	3.5	2.4×10^2	7×10^4
Exp. 2	35	70	75	2.1	2.3×10^2	8×10^4
no DNA	<2	<2	<2	-		-

Table 2 shows that these pooled transfectants secrete significant levels of growth hormone. Additionally, these transfected cells synthesize higher levels of growth hormone following induction with dexamethasone (dex) or with dex plus T$_3$ (Table 2). Thus the HPRT-GH-virus construct is capable of directing the synthesis of GH after transfection into cells.

The HPRT-GH-virus construct is incapable of synthesizing viral proteins, so helper virus is required to rescue this replication defective virus. It was attempted to rescue virus containing the HPRT and GH genes by superinfecting the transfected cells with Moloney murine leukemia virus (MoMLV). Both the HPRT-GH-virus and the MoMLV helper virus should be packaged into virions by proteins synthesized by the helper virus. Table 2 shows that infectious virus transmitting the HPRT$^+$ phenotype is indeed rescued from the transfected, MoMLV superinfected cells. The ratio of HPRT-GH-virus to helper virus is similar to that previously reported for the parent HPRT-virus, Miller et al., Proc. Natn. Acad. Sci. USA 80, 4709-4713 (1983), indicating that the presence of the GH minigene does not affect virus rescue.

To determine whether the GH minigene was being cotransferred in the rescued virus along with HPRT,

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virus rescued from cells transfected with pLPGHL or
pLPHGL was used to infect 208F rat cells and BALB 3T3
mouse cells. 16 independent HPRT$^+$ clones were
isolated and characterized for GH production. Because
5   GH is secreted from cells, it provides a convenient and
sensitive means to monitor the expression of the
transferred gene. Table 3 shows that of the 8 HPRT$^+$
rat clones and 8 HPRT$^+$ mouse clones, at least half
synthesize significant amounts of GH. In the remaining
10   infectants, either the minigene is inactive or failed to
co-transfer with the HPRT gene.

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TABLE 3

GH PRODUCTION AND INDUCTION IN CELLS INFECTED WITH VIRUS RESCUED FROM TRANSFECTED CELLS

Rescued virus	HPRT+ clone number	Growth hormone production			
		Control	dex	dex+T$_3$	Fold-Induction

A. 208F Rat Cell Infectants

Rescued virus	HPRT+ clone number	Control	dex	dex+T$_3$	Fold-Induction
LPGHL	F0.2	6.0	22	30	5.0
	F0.3	6.5	7.9	8.9	1.4
	F1.2	13	20	20	1.5
	F1.3	3.1	2.4	2.7	(0.9)
LPHGL	F2.1	3.3	3.6	2.9	(0.9)
	F2.4	4.0	3.7	2.4	(0.6)
	F3.2	25	93	126	5.0
	F3.3	4.5	4.4	4.0	(0.9)
uninfected cells		<1.5	<1.5	<1.5	-

B. Balb/3T3 Mouse Cell Infectants

Rescued virus	HPRT+ clone number	Control	dex	dex+T$_3$	Fold-Induction
LPGHL	B0.2	12	10	12	1.0
	B0.4	12	10	16	1.3
	B1.2	3.5	7.60	8.6	(2.5)
	B1.4	2.7	5.9	6.8	(2.5)
LPHGL	B2.3	<1	<1	<1	-
	B2.4	<1	<1	<1	-
	B3.3	236	246	289	1.2
	B3.4	<1	<1	<1	-
uninfected cells		<1	<1	<1	-

*GH production from induced or uninduced cells was measured as described in Table 1. HPRT+ colonies induced by virus rescued from transfected 208F cells (Table 1) were isolated using cloning cylinders. The first letter of the clone designation refers to the cell type of the infectant (F for 208F rat cells or B for Balb/3T3 mouse cells). Fold-induction values which may not be significant, due to limitations of the assay at low GH levels, are indicated in parentheses.

The endogenous GH gene in cultured pituitary cells and in the animals has been shown to be transcriptionally regulated by both glucocorticoid and thyroid hormones, although the potential sequences

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involved in this regulation have not been localized
Evans et al., Proc. Natn. Acad. Sci. USA 79: 7659-7663
(1982), Spindler et al., J. Biol. Chem., 257:
11627-11632 (1982). Two of the rat infectants (clones
5   FO.2 and F3.2 Table 3A) are clearly responsive to dex,
and this response is potentiated by T$_3$. Expression
and regulation appears to be independent of the
transcriptional orientation of the GH minigene. In
addition, two of the mouse clones appear to respond to
10 dex and T$_3$ (clones B1.2 and B1.4, Table 3B), although
the levels of GH production are low.

    Southern blot analysis provides an alternative
approach to follow the propagation of infectious virus
containing both HPRT and the GH minigene that is not
15 dependent upon expression. Therefore the DNA structure
of the integrated viruses was examined in several of the
infectants. Retroviruses integrate into cellular DNA in
a linear fashion and are flanked by LTRs. Thus, the GH
minigene should always be flanked by viral LTRs although
20 the integration site in the cellular genome is likely to
be random. DNA extracted from several viral infectants
was cleaved with the restriction enzyme which cuts only
once in the HPRT-GH-provirus. The resulting fragments
were analyzed by the technique of Southern, Southern E.
25 M., J. Mol. Biol. 98, 503-517 (1975), using a
radio-labelled GH probe. The GH probe hybridizes to a
single band in uninfected mouse and rat cells,
presumably derived from the endogenous GH gene, while in
DNA prepared from five infectant clones an additional
30 band was detected. The DNA fragment containing the
HPRT-GH-provirus has a different size in each of the
infectants, as expected, due to the random occurrence of
BamHI sites in the cellular DNA near the virus integra-
tion site. The results also show that each infectant
35 contains a single integrated provirus, as only one new
band is present in DNA from each of the clones. Similar
results were obtained using HPRT sequences as a probe.

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Finally, DNA from these clones was digested
with SstI and subjected to the same analysis. SstI cuts
once in each LTR of the virus and should reveal the size
of the integrated HPRT-GH-provirus. In DNA from each of
5   the infectants, there is a single new band in addition
to the endogenous band. As expected, the new bands have
the same size and all match the expected size (5.3 kb)
of an integrated HPRT-GH-provirus. Thus, no major
rearrangement of the viral genome has occurred during
10   generation of virus or of infection of these clones. It
is concluded that the simultaneous propagation of HPRT
and the GH minigene is independent of transcriptional
orientation of the later relative to the viral LTR.

       To examine transcription from the
15   HPRT-GH-provirus, combinations of RNA blot and S1
nuclease analysis were performed. Northern blot
analysis of growth hormone mRNA synthesized by the
integrated virus in several infected cell lines was
performed. The major mRNA species produced in all of
20   the infectants is the same size (1kb) regardless of the
transcriptional orientation of the minigene.
Furthermore, this transcript is identical to the RNA
product of the endogenous GH gene in rat pituitary
cells. S1 nuclease analysis was used to characterize
25   the 5' terminus to determine whether the appropriate GH
transcriptional signals were being utilized. The
results show that mRNA produced from the endogenous GH
gene protects a 189 nt fragment spanning the promoter
region and identifies a 5' cap site 25 nt from the
30   sequence TATAA. An identical size fragment is protected
by mRNA obtained from HPRT-GH-virus infected mouse or
rat cells, thus confirming accurate initiation from the
minigene. Furthermore, S1 nuclease and mRNA size
analysis imply that the GH polyadenylation signals are
35   being used.

       RNA analysis confirms that the GH minigene, in
addition to containing functional initiation and

polyadenylation sites, also remains inducible with dex
and T3. mRNA induction parallels increases in GH
production (Table 3). For example, rat clone F3.2
(Table 3A) exhibits a 5-fold induction of both GH mRNA

5　and secreted protein following induction. Clone F0.2
exhibits similar changes in both RNA and GH synthesis.
On the other hand, mouse clone B3.3 (Table 3B)
synthesizes constituitive and relatively high levels of
GH, which remains unaffected by hormone administration.

10　Correspondingly high GH mRNA levels also remain
unaltered. Some clones, such as B1.4 and F1.2, reveal
low levels of both GH RNA and secreted protein.

Although in this example the mini-gene was
assembled from a cDNA that encodes the rGH protein and

15　from the rGH promoter sequence, it is to be understood
that the gene-encoding sequence may be linked to and
under the translational control of a promoter sequence
to which it is not linked in nature. A gene-encoding
sequence, for example, might be linked to a promoter

20　sequence derived from another species, particularly
where a protein endogenous to one species is to be
raised in the cells of another species.

The invention, as exemplified by the above
examples, substantially expands the utility of

25　retroviruses as vectors for carrying genetic information
into foreign cells. Through the use of helper viruses
encoding amphotropic packaging proteins, recombinant
retrovirion are generated which are generally useful for
carrying genetic information into vertebrate cells.

30　Novel defective helper viruses are provided for
generating recombinant retrovirions that are useful for
a one-time infection and that are free of infectious
helper virus. Unique constructs are provided
incorporating proviral LTR sequences but deleting

35　sequences, e.g., poly A sequences, which tend to limit
transcription, thereby improving the utility of the
retroviral vectors for carrying and expressing foreign

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DNA sequences.

Further provided by the invention are cells
that have been genetically altered by recombinant
retroviral vectors and are shown to continue to express
5   gene-product after the cells are transplanted into a
living animal. Retroviral vectors, including
helper-free vectors, are shown to genetically alter
somatic cells of a living animal by the most simple
techniques, such as directly infecting the animal
10 intraveneously with a suspension of recombinant
retroviral vectors. Both implantation of genetically
altered cells and genetic alteration of somatic cells by
direct infection are important gene therapy tools.

Foreign genes may be inserted into retroviral
15 constructs either under the promotional control of the
strong viral LTR promoters or under the control of
linked non-viral promoter sequence. Both types of
promotional control have advantages in particular
situations.

20 While the invention has been described with
reference to certain preferred embodiments,
modifications obvious to one with ordinary skill in the
art may be made without departing from the scope of the
present invention.

25 Various features of the invention are set forth
in the following claims.

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1.   In combination, A) a recombinant construct
comprising a 5' retroviral LTR nucleotide sequence
including a cell genome insertion site, a genome
packaging sequence linked to said 5' LTR sequence, a
5   promotor sequence, a heterologous protein-encoding
sequence under promotional control of said promoter
sequence and a 3' retroviral LTR sequence including a
cell genome insertion site, said construct being
deficient in viral protein-encoding sequences needed to
10 package the corresponding RNA retroviral genome as a
complete retrovirion; and B) a helper retroviral vector
useful for rescuing the retroviral genome corresponding
to said recombinant construct, said helper retroviral
vector comprising sequences encoding the requisite
15 packaging proteins for packaging said retroviral genome,
said packaging protein-encoding sequences of said helper
retroviral vector including a sequence that encodes an
amphotropic envelope protein, and a promoter sequence or
sequences linked to said packaging protein-encoding
20 sequences for expression of packaging proteins, said
helper retroviral vector being deficient in its own
packaging sequences.

2.   A combination in accordance with claim 1
wherein said recombinant construct encodes no viral
25 packaging proteins and wherein said helper retroviral
vector encodes the full complement of proteins necessary
for packaging said retroviral genome.

3.   A helper retroviral vector being useful
for rescuing a retroviral genome corresponding to a
30 recombinant DNA construct that is deficient in packaging
protein-encoding sequences, said helper retroviral
vector comprising nucleotide sequences encoding the
requisite packaging proteins that are lacking from the
construct for packaging the retroviral genome, said
35 packaging protein-encoding sequences of said helper

retroviral vector including a sequence that encodes an
amphotropic envelope protein and a promoter sequence or
sequences linked to said packaging protein-encoding
sequences for expression of packaging proteins, said

5   helper retroviral vector being deficient in its own
packaging sequences.

        4.   A helper retroviral vector in accordance
with Claim 3 useful for rescuing a retroviral genome
corresponding to a recombinant DNA construct that

10   encodes no packaging proteins, said retroviral vector
having sequences that encode the full complement of
proteins necessary for packaging said genome.

        5.   A titer of infectious retrovirions, which
retrovirions each comprise a recombinant genome having a

15   5' LTR sequence including a cell genome insertion site,
a promoter sequence, a heterologous protein-encoding
sequence under the promotion control of said promoter
sequence and a 3' retroviral LTR sequence including a
cell genome insertion site, said genome being deficient

20   in viral protein-encoding sequences needed to package
progeny genomes as complete retrovirions, and a full
complement of packaging proteins packaging said genome
for said retrovirion to be infective of eukaryotic
cells, said packaging proteins of each retrovirion

25   including an amphotropic envelope protein, whereby said
recombinant retrovirions are infective across biological
families, said packaging proteins being provided by a
helper retroviral vector to the extent that said genome
is deficient in viral packaging protein-encoding

3.0 sequences, said titer being free of helper retroviral
vector.

        6.   A titer according to Claim 5 wherein said
genome encodes no retroviral packaging proteins and all
of said packaging proteins are provided by the helper

35   retroviral vector.

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7. A titer according to Claim 6 wherein said
retrovirions have amphotropic envelope proteins which
make said retrovirons infective of human cells.

8. A method of imparting a genetic

5 characteristic to a selected eukaryotic cell through a
retrovirion infection comprising assembling a
recombinant DNA construct comprising a 5' viral LTR
sequence including a cell genome insertion site, a
packaging sequence, a promotor sequence, a heterologous

10 protein-encoding sequence under the promotional control
of said promoter sequence and a 3' viral LTR sequence
including a cell genome insertion site, said construct
being deficient in viral protein-encoding sequences
needed to package the corresponding retroviral genome as

15 a complete retrovirion, including being deficient an
envelope protein-encoding sequence, providing a helper
retroviral vector having nucleotide sequences encoding
the viral packaging proteins, including an amphotropic
envelope protein, needed to package said retroviral

20 genome and sequences promoting expression of said viral
packaging protein-encoding sequences, said helper
retroviral vector being deficient in its own packaging
sequence, introducing said construct into a host
eukaryotic cell, rescuing the genome corresponding to a

25 said construct as an infectious retrovirion from said
host cell with said helper retroviral vector, and
infecting said selected eukaryotic cell with said
retrovirion.

9. A method according to claim 8 wherein said

30 host eukaryotic cell and said selected eukaryotic cell
are from species of different biological families.

10. A method according to Claim 9 wherein said
host eukaryotic cell is a murine cell and said selected
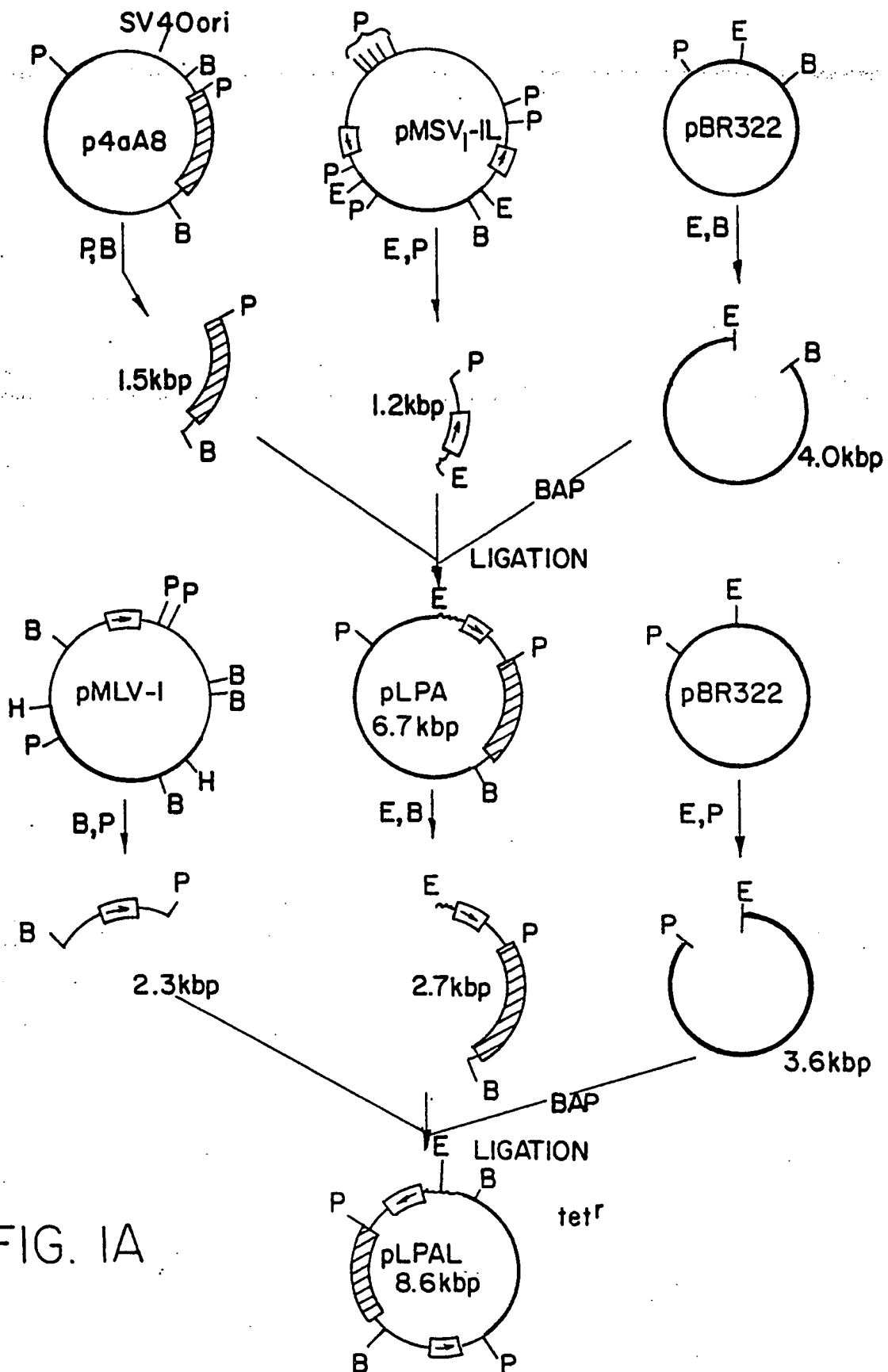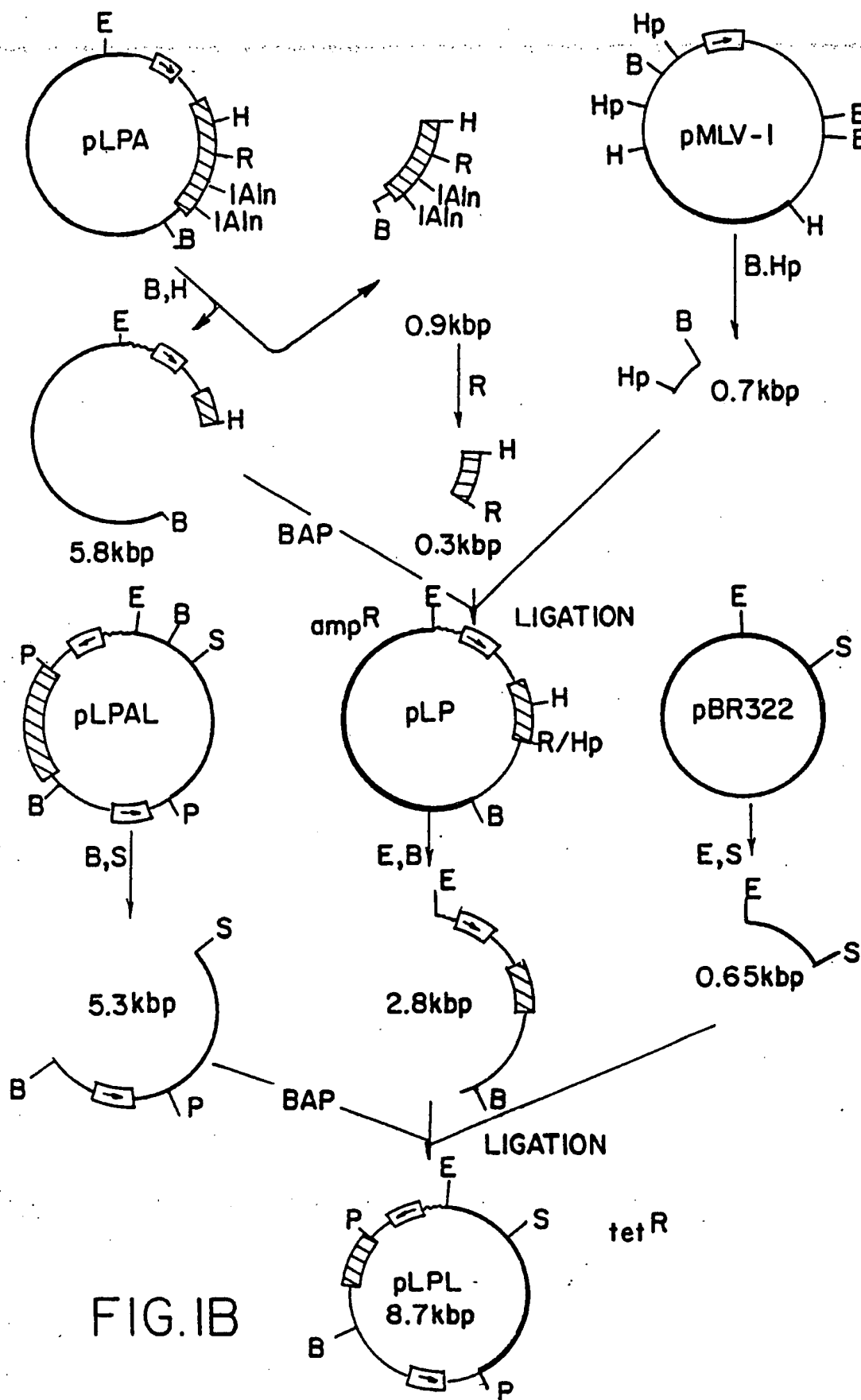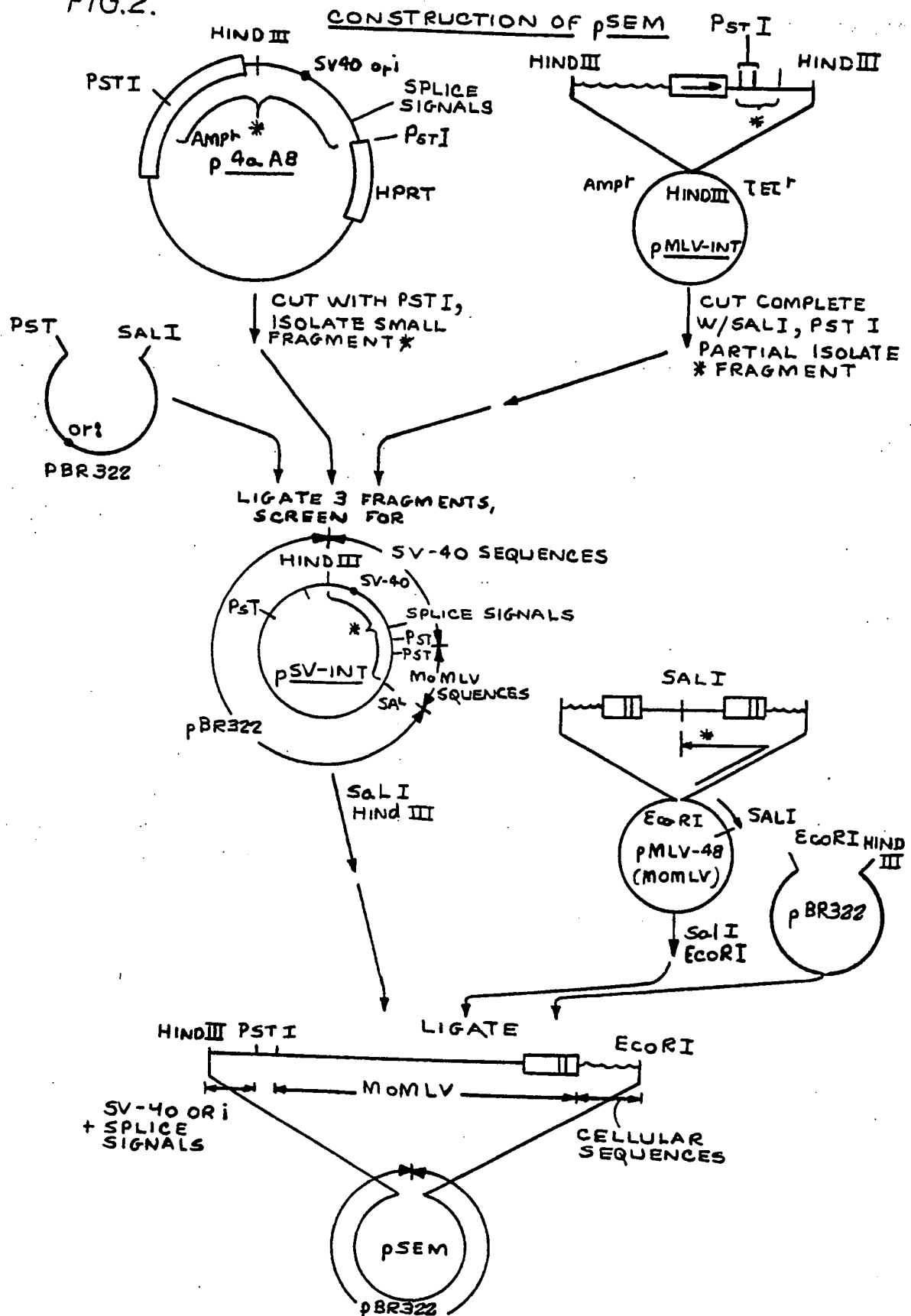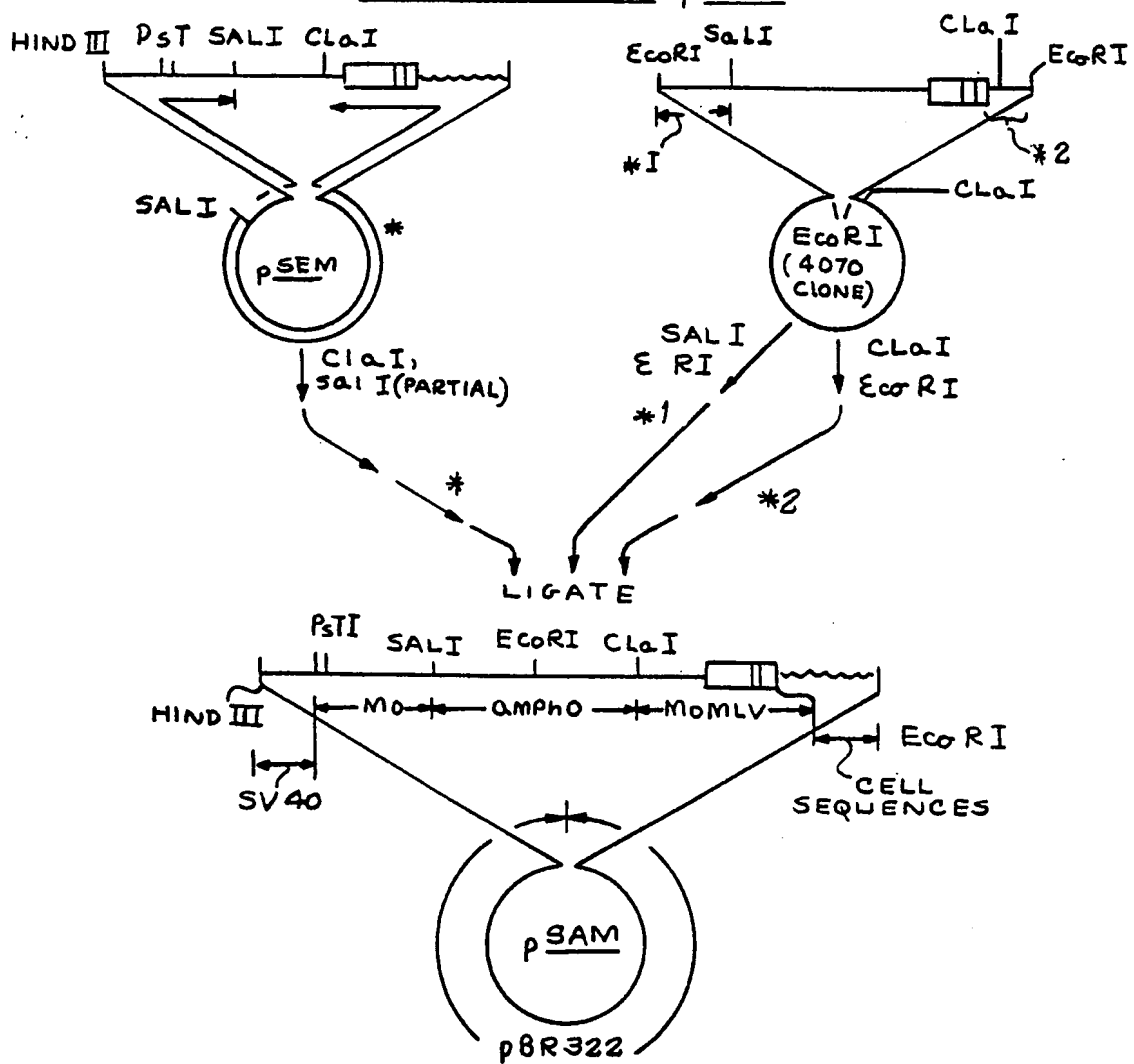eukaryotic cell is a human cell.

FIG. IA

FIG.1B

FIG.2.   CONSTRUCTION OF pSEM

FIG.3.   CONSTRUCTION OF pSAM

FIG 4A

FIG.4B.

GH MINI GENE
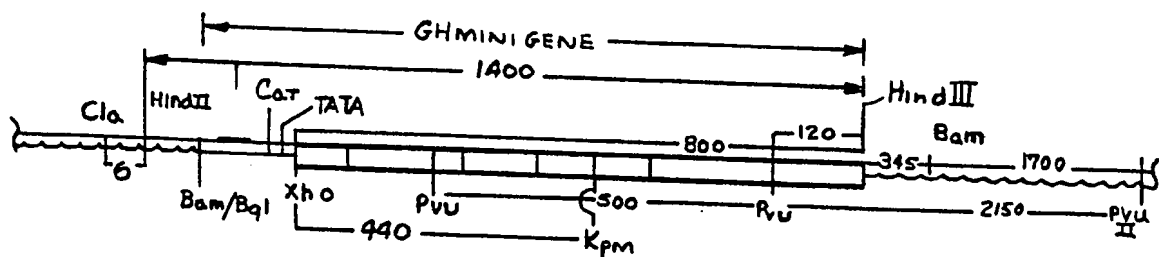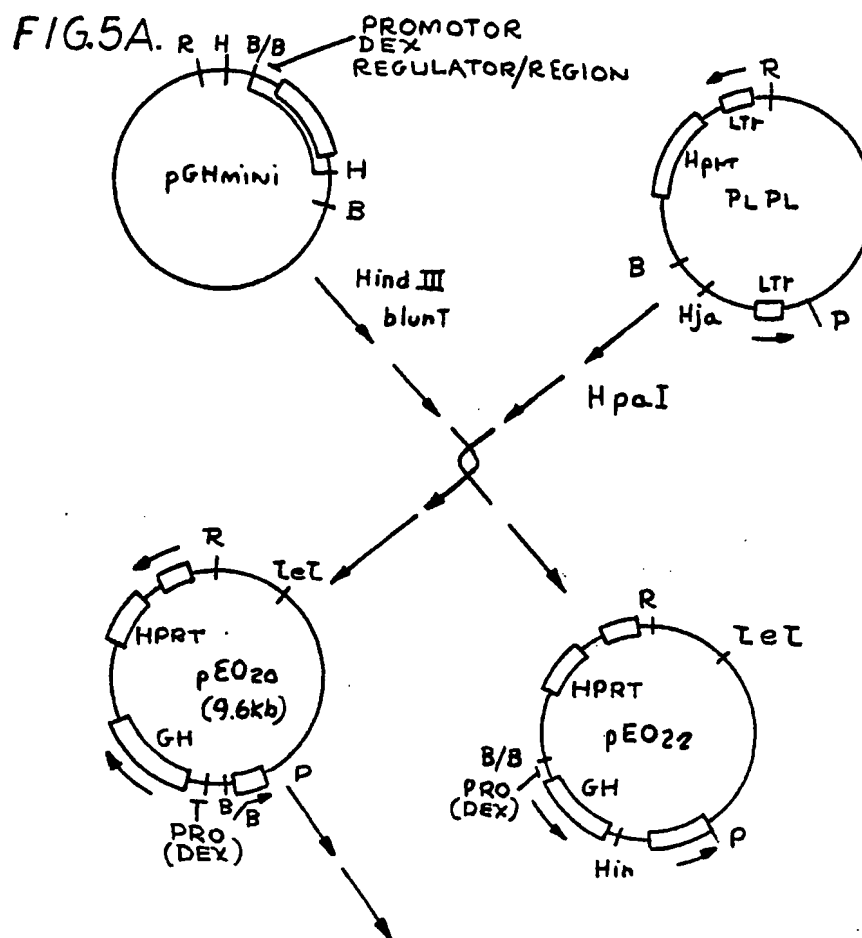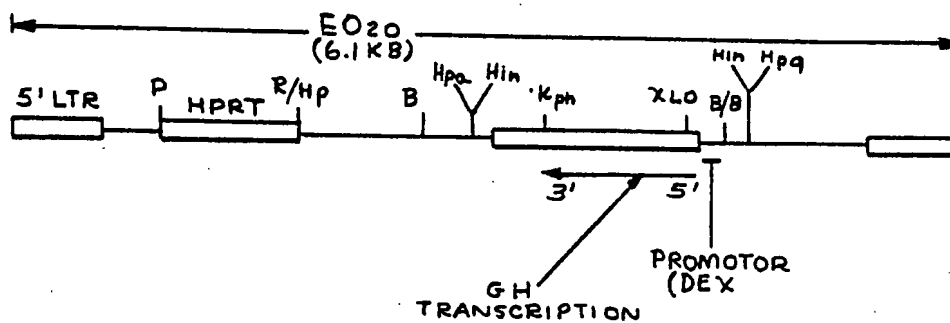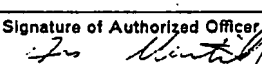
FIG.5A.
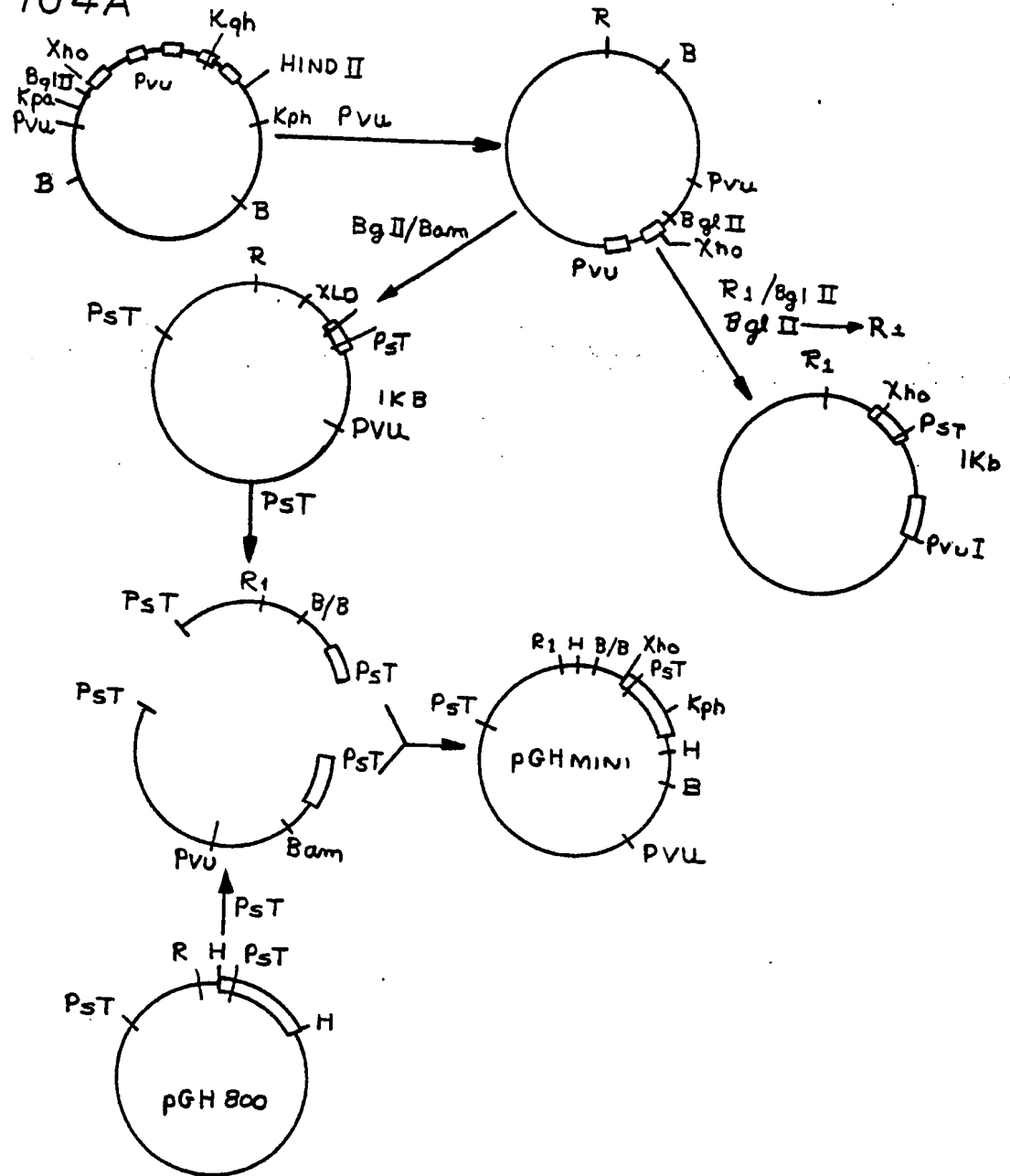
FIG.5B.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01442

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC: [4] C12N 1/00, C12N 7/00, C12N 7/04, C12N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68,70,71,91,172.3,235,236,239,317; 536/27; 424/89

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [5]

CA SEARCH DATABASE: 1977-85

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with Indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US,A, 4,405,712 Published 20 September 1983, Vande Woude et al.	1-10
X	N,Willis et al, J.Biol.Chem., Vol. 259, June 1984, pages 7842-7849	1-10
Y	N,Brennand et al, J.Biol.Chem., Vol.258, August 1983, pages 9593-9596.	1-10
Y	N,Tabin et al, Molecular and Cellular Biology, April 1982, pages 426-436	1-10

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [3]
02 October 1985	10 OCT 1985
International Searching Authority [1]	Signature of Authorized Officer
ISA/US	James Martinell

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
Y	N,Shimotohno et al, Cell, Vol 26, 1981, pages 67-76	1-10
Y	N,Wei et al, J.Virol., Vol. 39, September 1981, pages 935-944	1-10
Y,P	N,Anderson, Science, Vol. 226, October 1984, pages 401-409	1-10

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers............, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers............, because they relate to parts of the International application that do not comply with the prescribed require-ments to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this International application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	N,Miller et al, Proc. Natl. Acad. Sci. USA, Vol.80, August 1983, pages 4709-4713	1-10

Form PCT ISA.210 (extra sheet) (October 1981)